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THE PRIMARY SEQUENCE OF ACETYLCHOLINESTERASE
AND SELECTIVE ANTIBODIES FOR THE DETECTION
OF ORGANOPHOSPHATE TOXICITY

ANNUAL/FINAL REPORT
PALMER TAYLOR

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<p>The proposed work has been directed to determination of the structure of acetylcholinesterase. During the project period we have determined the primary structure of the Torpedo enzyme through amino acid sequencing and the isolation and sequencing of a cDNA clone encoding for the 11S form of the enzyme. The recombinant DNA studies involving the isolation of cDNA clones encoding acetylcholinesterase were part of studies supported by an NIH grant. However, they relied on amino acid sequence information for preparation of oligonucleotides for screening and ultimately yielded complementary information on primary structure. Peptides corresponding to the active center of the enzyme and a C-terminal region have been synthesized and antibodies are being raised for the purpose of detecting the phosphorylated enzyme and delineating functional regions of the molecule. Finally, the disulfide bonding pattern has been determined for the 11S form of the enzyme.</p>					
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SUMMARY

The proposed work has been directed to determination of the structure of acetylcholinesterase. During the project period we have determined the primary structure of the Torpedo enzyme through amino acid sequencing and the isolation and sequencing of a cDNA clone encoding for the 11S form of the enzyme. The recombinant DNA studies involving the isolation of cDNA clones encoding acetylcholinesterase were part of studies supported by an NIH grant. However, they relied on amino acid sequence information for preparation of oligonucleotides for screening and ultimately yielded complementary information on primary structure. Peptides corresponding to the active center of the enzyme and a C-terminal region have been synthesized and antibodies are being raised for the purpose of detecting the phosphorylated enzyme and delineating functional regions of the molecule. Finally, the disulfide bonding pattern has been determined for the 11S form of the enzyme.



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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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Introduction

During this period we have completed the primary structure of acetylcholinesterase from Torpedo californica through amino acid sequencing and cDNA cloning and sequencing. This information should prove important to the many groups working on acetylcholinesterase structure.

Background

The high turnover number of acetylcholinesterase and the availability of selective inhibitors have allowed extensive study of its distribution, catalytic mechanism, and functional role in cholinergic neurotransmission. The recent elucidation of the primary structure of the enzyme through protein chemistry and the isolation of its gene have added a molecular dimension to continuing studies on this protein, which controls the residence time of acetylcholine in the synapse. Acetylcholinesterase exhibits an extensive polymorphism of structure and, since the catalytic parameters of the individual enzyme species are largely invariant, the structural diversity appears critical only to the regulation of the cellular disposition of this molecule. Recent structural studies clearly show that acetylcholinesterase behaves as a secreted rather than an integral membrane protein. The posttranslational modifications provide an appropriate link to tether the enzyme to specific extracellular locations. Since acetylcholinesterase is an extracellular enzyme, modifications of structure critical to its disposition should occur prior to export to its site of residence. Thus variations in structure responsible for cellular localization must either be encoded in the genome or be differentially affected by post-translational events of biosynthesis.

Acetylcholinesterase Polymorphism

Since the initial finding of Massoulié and Reiger (1) that a native form of acetylcholinesterase contains an elongated tail unit linked to a defined number of catalytic subunits, the control of individual species of acetylcholinesterase in relation to innervation, developmental processes and activity of excitable cells has received considerable attention (2). Two general classes of acetylcholinesterase species exist. The most unique is the elongated or dimensionally asymmetric species, which contains a filamentous tail unit disulfide-linked to tetrameric sets of catalytic subunits. The tail unit contains a collagen-like sequence distal to the catalytic subunits. Each strand of the triple helix is joined to a tetramer of catalytic subunits. Since each catalytic subunit is approximately 70,000 daltons, elongated species close to a molecular weight of 1 million are generated. In the case of Torpedo, but not Electrophorus, a second type of structural subunit has been identified as a noncollagenous, 100,000-dalton peptide (3). It will be of interest if this structural entity also prevails in higher species. Treatment of the asymmetric form with collagenase markedly shortens the tail unit and a light tryptic digestion will remove the structural subunits without apparently altering catalytic parameters or the structure of the catalytic subunit (cf. 2). The asymmetric species appear to be fully assembled in the Golgi apparatus prior to export from the cell (4,5).

The second class includes the globular forms, which show considerable structural variation in subunit assembly (monomers to tetramers) and in hydrophobicity. The hydrophobic forms identified to date result from the cotranslational addition of glycopospholipid to the C-terminal carboxyl group of the nascent peptide chain (6,7). This modification resembles that seen in the variable surface glycoprotein of trypanosomes and the Thy-1 antigen (8). It is quite possible that the natures of the glycopospholipid additions are not identical in the various tissues and may, in themselves, provide a basis for microscopic regional localization. Hence, the globular forms range from totally soluble species to species with particular hydrophobic glycopospholipids conjugated to the peptide chain.

Methods

The methods used for generation of the data described below have been documented in our manuscripts now published in the open literature (3,9-12) and will be described only briefly.

Our overall strategy was to sequence the catalytic subunits from the 11S and 5.6S forms of the enzyme. The primary peptides were obtained from a tryptic digest after disulfide bond reduction and carboxymethylation with iodoacetate. They were then size fractionated on Sephadex G-50. The individual fractions were categorized as I-X and then fractionated on reverse phase high pressure liquid chromatography (HPLC) using a C-18 column with 0.1% trifluoroacetic acid (TFA) and usually a 0-50% gradient of acetonitrile. These peptides were then numbered by consecutive fractions and catalogued. In cases where fractionations were incomplete, fractions were then subjected to HPLC using a C-4 column or HPLC on C-18 or C-4 columns using a phosphate, pH 7.0 buffer. Details on the cataloging of peptides and their sequences may be found in the year-end report (year 2) (13). As noted below, a similar strategy was employed to obtain the disulfide linked peptides.

The enzymes were purified to homogeneity by affinity chromatographic procedures adopted in our laboratory many years ago (3). Each enzyme form was homogenous, as ascertained by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and by assay of specific activity.

1. Determination of the primary structure of acetylcholinesterase

Primary structure determinations relied on both tryptic and CNBr fragmentation. The peptides were initially size-separated on Sephadex G-50 and then subjected to reverse-phase HPLC on C-18 or C-4 columns. Sequencing initially involved the dansyl-Edman and the spinning cup methods, but after the first 6 months of the contract we employed the gas phase method (9). The changeover to the gas phase method followed our making the instrumentation operational and obtaining reliable and reproducible data on representative peptides. Amounts down to 50 pmol of peptide could be sequenced by the latter method. Our basic sequencing strategy for the tryptic peptides was to reduce and alkylate the protein with [¹⁴C]iodoacetate and size fractions were collected which were then subjected to reverse-phase HPLC on C-4 columns, using an acetonitrile-1% aqueous TFA gradient. Peptides that fractionated poorly on C-18 columns usually resolved well on C-4 columns. Compositions and N-terminals were ascertained before subjecting the peptides to gas-phase sequencing. Cyanogenbromide (CNBr)

fractionations also involved reduction and alkylation with [¹⁴C]-iodoacetate, treatment with CNBr and size fractionation on Sephadex prior to HPLC. For the CNBr peptides, fractionation on C-4 columns worked best. In some fractionations the active site peptide was labeled with [³H]diisopropylfluorophosphate.

2. Antibody generation and assessment of reactivity

Both monoclonal and polyclonal antibodies were made to the 11S and 5.6S species of acetylcholinesterase. Similar methods were used for generation of antibodies to the individual peptides. Antibody reactivity and titers were determined by the enzyme-linked immunoassay (ELISA) method and by radioimmunoassay using [¹²⁵I]acetylcholinesterase (3,10).

3. Cloning and sequencing of a cDNA-clone encoding the 11S species of acetylcholinesterase

Nucleotide probes to tandem sequences contained within a single CNBr peptide were used to hybridize recombinants in a λ-gt 10 library (11). Positive clones were isolated, tested by hybridization and sequenced using M-13 sequencing vectors (11).

4. Determination of the disulfide bond linkages in the 11S species of acetylcholinesterase

Unreduced acetylcholinesterase was reacted with bromobimane to label free sulfhydryls. Tryptic and CNBr peptides were prepared and candidate disulfide peptides isolated by having unique positions on HPLC. These peptides were then reduced by dithiothreitol and alkylated with [¹⁴C]iodoacetate. Upon chromatography, these peptides were isolated and sequenced (12).

Results

1. Amino acid sequencing of Torpedo acetylcholinesterase

Our sequencing strategy is designed to fulfill several objectives:

a. To obtain a sequence sufficient for the design of multiple nucleotide probes for cDNA library screening.

b. To employ the sequence to verify inferred amino acid sequence resulting from cDNA sequencing and correlate the cDNA sequences with the multiple acetylcholinesterase gene products.

c. To elucidate differences in sequence between the molecular forms of acetylcholinesterase.

d. To identify critical regions in the molecule: active center, chemically modified residues, N-terminal sequence, C-terminal sequence, glycosylation sites, cysteine-containing peptides and other sites of post-translational modification.

e. To provide a peptide fractionation scheme by which other cholinesterases of lower abundance can be sequenced and homologous regions identified.

To date, we have sequenced about 80% of the tryptic peptides of the 11S enzyme and 30% of the tryptic peptides of the 5.6S enzyme. All of the CNBr peptides have been isolated from the 11S enzyme and about 30% have been sequenced. A smaller number have been sequenced in the 5.6S enzyme. The sequences are summarized in Table I. Several findings should be highlighted:

a. Large tryptic peptides for the active center (24aa) were isolated and sequenced. Sequence was verified by chymotryptic digestion and the position of this peptide in the whole enzyme could later be verified. The active center serine is serine 200. These peptides are identical in the 11S and 5.6S species (1) (Fig. 1).

b. The N-terminal peptides of the 11S and 5.6S enzymes were identified and sequenced through 42 and 30 residues, respectively. These peptides were also identical in the 11S and 5.6S species. In some cases glutamine appeared at position 6 and asparagine at position 3, but this occurred in the minority of cases. These sequences were later verified by the cDNA sequence and, more important, a leader peptide was demonstrated for the unprocessed acetylcholinesterase. Cleavage occurred C-terminal to an Ala, giving rise to the N-terminal Asp residue in the processed protein. A candidate C-terminal tryptic peptide ending in leucine was also identified in the 11S species. That this peptide was a C-terminal tryptic peptide was later verified by finding a stop codon corresponding to amino acid position 575, which followed the leucine codon and thus ended the open reading frame on our cloned cDNA. An analogous C-terminal peptide has not been found for the 5.6S enzyme, and we believe a posttranslational modification occurred here, providing one of the points of structural departure of the two enzyme forms.

c. The cysteine-containing peptides were identified by reduction and subsequent alkylation by [¹⁴C]iodoacetate. We obtained more cysteine peptides than would be predicted by the c-DNA sequence, but they arose simply from incomplete cleavages. All of these peptides can be placed in the inferred amino acid sequence on the basis of either their total sequence or their N-terminal residue identification and partial sequences. We have initiated fractionations of the unreduced enzyme with the essential aim of establishing the positions of the inter- and intrasubunit disulfide bridges. One of the eight cysteines appears to exist as a free sulfhydryl group and

Table I Sequences of Torpedo californica Acetylcholinesterase Peptides*

<u>11S Acetylcholinesterase</u> <u>(tryptic peptides)</u>		<u>11S Acetylcholinesterase (tryptic</u> <u>peptides cont'd)</u>	
I46	ivgywapfa-c	V187	vq-cwfnqflp
I7	vpvegcvfane-f-nnci	VIII7	rpepk
III88	fsivpvddggfw(yst)k	V01	tgnpnephsqesk
II61	kpwsgvw-asnyp (carbohydrate and CM cysteine)	V02	fidlntepmnk
II61	kpwigvwfhnypl	V03	ailqsgspncpwasvsv
IV33	dnhellvntksgkvmgrvpvlsshisaf1 givfaeqvgidv (N-terminal)	V04	galqvwhdniqffggdpmk
IV67	tvtfifgesaggasvghilspgsr (active site)	III65	--dedcly-niw-pgc
IV14	tgnpnepstqesk	II61	pw-gv--a--vpl
IV26	le-ea	IV26	hescael (C-terminal peptide)
IV33	ailqsg-vdcepa	I77	v-vegcvfane-nnci
I46	ivgywaafa-c	II01	d-nlvwpew-gvi-gy
I77	vpvegcvfane-f-nnci	II49	dlbbglncl-nsaeeeli--cl
V187	vqvcwfnqflp	II59	lgvpda
VIII7	rpepk	II67	l-vphandlgldtvg!qytdwmd
V01	fidlntepmnk	III88	f-ivpv-dgqfw
V02	galqvwhdniqffggdpmk	IV14	tgnpnepstqe
I67	iteahh	IV62	fgdgtly
II49	nlbbglncl-nsaeeelihcl	IV64	aieag
III65	-(av)dedcly-niwspgca	IV71	ivtifg-s
IV69	v-afalig	I46	ivgywa-fa
IV67	vphandlgldv(g)lqytdwmdnngik	I67	iteah
IV26	hescael (C-term)	IV69	v-afali
II61	kpw(i)gvw-as(n)ypl (carbohydrate, CM cysteine)	<u>5.6S Acetylcholinesterase (tryptic peptide)</u>	
I46	ivgywa2fa-(c)	IV18	tgnpnep
I77	v(p)vegcvfane(f)(lp)nnci	IX02	gpha-a
III88	f(s)ivpv(d)dgqfw(ystk)	VI64	ail-e--pncpwwatv-va
II63	dglddivgbhnicplmhf		dnhqllvntksgkvmgt (N-terminal)
II62	kpwphawdlg-p		tvtfifgesaggasvghilspgsr (active site)
II64	lsvphandlgldlt		dnhsqllvntksgkv-gt (N-terminal)
II101	dhnlvwpew-gvi(h)gyei--g-l-p	IV67	tvtfifg
II68	lsvphandlgldtvgloytdwmd(ing) (4-E7 immunoreactive)	II63	dglddivgdhnicplmhf
I77	v(aph)vegcvfane(yf)(lp)(np)nc(f)- (hg)v(if)e	II64	vphandlgld(dw)avt
II49	nlbbglncl-nasagglhihcl (carbo)	VI64	a(i)lqsgsp(ns)cpwatv-va
III69	-(av)(sd)edcly-niw(s)pgca	IV62-64	fgbgtyly(f)(f)n(h)r
IV33	ailqsgspncpwasvsv(aZg)p	IV18	tgnpnep(p)vzeq
V187	vg(v)cxfwngflp	IX02	gpha-a
I61	kpw(l)gvw(f)(h)(n)y(p)l	IV64	aigag(a)(v)ae(pg)g-(v)-ppd
I77	v-vegcvfane--ncf--v(ip)g	II54	lg(v)(p)s(la)a--(dv)
III64	b dedcly-niw-pgc	II09	vdl(l)
V03	ailqsgspncpwasvsv(azg)r	II59	lgvpda----d--vp
V01	tgnpnephsqesk	VI64	a-lqsg(s)pncpw
II54	lgvp-a	II09	(te)vdl(l)
IV64	aieag	II59	lgvpda----d--vp
IV71	ivtifg-s	VI64	a-lqsg(s)pncpw
		II67	lgvphad-dg
		VI64	ailqsgsp(ds)cpwatv-ia

11S Acetylcholinesterase (CNBr peptides)

*Roman numerals denote Sephadex fractions of the initial fractionation and Arabic numbers denote the peak fraction from high-pressure liquid chromatography. CM-cysteine denotes radioactivity associated with the carboxymethyl group, indicating that the peptide may contain cysteines; carbohydrate indicates carbohydrate-containing peptides. Parentheses indicate uncertain sequences and dashes indicate an undetectable amino acid appearing at that position. Peptides lacking identification codes were refractionated to achieve greater purity prior to sequencing.

$\begin{array}{c} \text{I}_2 \\ \text{ALA} \end{array} \rightarrow \text{Glu} \rightarrow \text{Val} \rightarrow \text{Ile} \xrightarrow{\text{I}_2} \text{Ile} \rightarrow \text{Gly} \rightarrow \text{Gly} \rightarrow \text{Ala} \xrightarrow{\text{I}_2} \text{Ala} \rightarrow \text{Ser} \rightarrow \text{Val} \rightarrow \text{Gly} \rightarrow \text{Met} \rightarrow \text{His} \rightarrow \text{Ile} \rightarrow \text{Leu} \rightarrow \text{Ser} \rightarrow \text{Pro} \rightarrow \text{Gly} \rightarrow \text{Ser} \rightarrow \text{Arg}$

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has been labeled with monobromobimane in the 5.6S enzyme. Isolation and sequencing of the peptide reveals that the cysteine is at position 231

Sites of glycosylation have been identified by lectin blotting of the individual peptides and by broad elution profiles that reflect microscopic heterogeneity within the peaks and their coalescence following endoglycosidase F treatment. An example is shown in Fig. 2. Three of the four potential N-linked glycosylation sites have been located by peptide isolation (asparagine positions 59, 457, and 533), while it appears that position 416, despite the presence of an Asn, X, Ser/Thr, is not glycosylated. Overall carbohydrate compositions suggest that we may have an O-linked site, but this remains to be established.

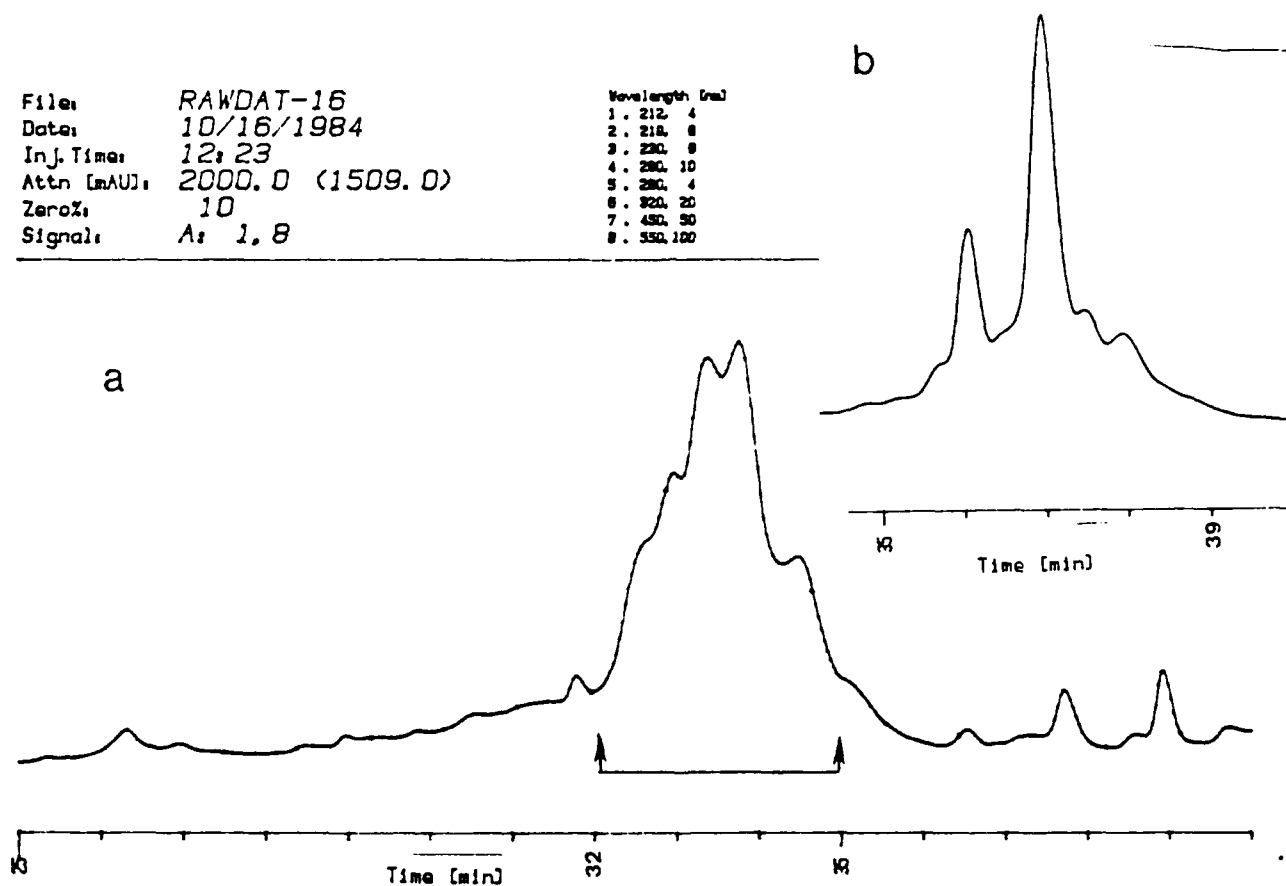


Figure 2: HPLC profiles of 11S acetylcholinesterase peptides prior to (a) and following (b) endoglycosidase F treatment. Fractions 32-35 were isolated, lyophilized and treated with endoglycosidase F. The fractions were run on the same column (C-18 reverse phase), using an identical trifluoroacetic acid-H₂O-acetonitrile gradient. The elution profile with its altered elution positions and decreased complexity is shown in the inset (b).

d. Potential sites that serve as epitopes for the monoclonal antibodies raised by B.P. Doctor (10) have also been identified. The two of particular interest are 4E-7 and AE-2. 4E-7 reacts selectively with the hydrophobic 5.6S enzyme (2) and has been found to react only with the glycosylated form of the enzyme. Treatment with endoglycosidase F but not endoglycosidase H eliminates the antigenicity. 4E-7 reacts equally well with the native and denatured enzyme. A peptide extending between residues 358 and 386 shows the greatest reactivity with 4E-7 as determined by antibody blotting and competitive immunoprecipitation. We expect this peptide to be one of those unique to the 5.6S enzyme.

The other antibody of interest is AE-2, an antibody isolated by Fambrough and colleagues (14) which shows considerable species cross-reactivity. AE2 was found to react with a peptide found by B.P. Doctor in fetal calf serum acetylcholinesterase. This peptide has been found between positions 12 and 18 and considerable homology between species exists in a large portion of this peptide (Fig. 3). Several other antibodies are less well characterized. However, some, such as 4G-7 and 2C-9, show high titers and good immunoprecipitation capacity.

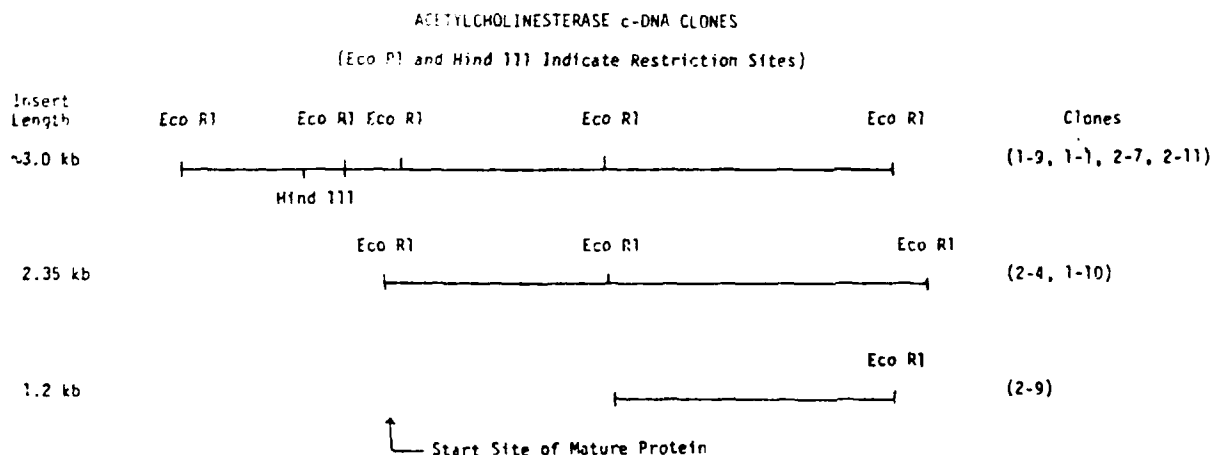


Figure 3: Characterization of several clones encoding for acetylcholinesterase. The length of the clones and their Eco RI and Hind III sites are shown.

The rather brief description given here describes the bulk of the studies performed during the past 2 years. Extensive fractionation and sequencing were required to achieve this state of progress for an enzyme subunit size of 575 amino acids which exists in multiple enzyme forms. These endeavors have been very much facilitated by the instrumentation provided in the contract. Profiles of some of the many fractionations can be found in MacPhee-Quigley et al. (9).

2. Preparation of antibodies directed to the active center for acetylcholinesterase

Having obtained the active center peptide sequence, we then synthesized a 25-mer peptide to generate antibodies to the active center of acetylcholinesterase. The peptide was synthesized by the Merrifield solid-phase method, using Dr. Russell Doolittle's facility, Department of Chemistry, University of California, San Diego. An N-terminal lysine was added to promote solubility, giving the sequence:

Lys-Thr-Val-Thr-Ile-Phe-Gly-Glu-Ser-Ala-Gly-Ala-Ser-Val-Gly-Met-Ile-Leu-Ser-Pro-Gly-Ser-Arg.

Antibodies are being generated in three ways: Monoclonals are being raised by B.P. Doctor and M.K. Gentry at Walter Reed Army Institute of Research. The fusions are now complete, positive colonies have been selected, and we should be screening for precise titers and selectivity next month. Polyclonal antibodies are also being raised in rabbits at San Diego, using two forms of immunogens: the peptide dispersed in liposomes and the peptide conjugated to hemocyanin. The monoclonal antibodies have the potential of being directed to small peptidic domains, some of which will show little species cross-reactivity. Others, owing to extensive homology in the epitope, will exhibit considerable species cross-reactivity. The polyclonal antibodies can be expected to have the higher titers and will prove most useful for screening in vitro translation products and the development of highly sensitive assays for the active center of acetylcholinesterase. Antibodies to synthetic peptides have the advantage of not showing cross-reactivity to contaminant proteins in biological preparations.

3. Comparative sequencing of Torpedo and other cholinesterases

Our initial findings showing extensive homology of the active center peptides of Torpedo acetylcholinesterase and human butyrylcholinesterase (Table II) and the substantial homology in the N-terminal region of the two proteins (Table III) prompted a further homology search in conjunction with Drs. Oksana Lockridge and Bert LaDu at the University of Michigan, and we see extensive similarity throughout the two molecules. Several peptides showing corresponding sequences can readily be found if our peptides (Table I) and their peptides are compared. The Michigan group also has about 80% of the peptides sequenced and with a total inferred sequence available in Torpedo, it should be possible for them to place the remaining peptides within the linear sequence (15).

Table II

Sequences of Active Site Regions

	5	P	10	15	20
TORPEDO ACETYLCHOLINESTERASE	NH ₂ - THR VAL THR ILE PHE GLY GLU SER ALA GLY GLY ALA SER VAL GLY MET HIS ILE LEU SER				
EEL ACETYLCHOLINESTERASE		GLY GLU SER SER GLU GLY ALA ALA GLY			
HUMAN PSEUDOCOLINESTERASE	NH ₂ - SER VAL THR LEU PHE GLY GLU SER ALA GLY ALA ALA SER VAL SER LEU HIS LEU LEU SER				
EQUINE PSEUDOCOLINESTERASE		PHE GLY GLU SER ALA GLY SER ALA ALA			
EQUINE ALTESTERASE		PHE GLY GLU SER ALA GLY ALA ALA SER			
BOVINE TRYPSINOGEN	LYS ASP SER CYS GLN GLY ASP SER GLY GLY PRO VAL VAL CYS SER GLY LYS				
PORCINE TRYPSIN	LYS ASP SER CYS GLN GLY ASP SER GLY GLY PRO VAL VAL CYS ASN GLY GLN				
S. GRICEUS TRYPSIN	VAL ASP THR CYS GLN GLY ASP SER GLY GLY PRO MET PHE ARG LYS ASP ASN				
E. COLI ALKALINE PHOSPHATASE	LYS PRO ASP TYR VAL THR ASP SER ALA ALA SER ALA THR ALA TRP SER THR				

Human butyrylcholinesterase and Torpedo acetylcholinesterase can be expected to diverge on a phylogenetic basis and the basis of distinct enzymatic properties (i.e., the butyrylcholinesterase will accommodate substrates with large acyl groups, it does not show substrate inhibition and it is preferentially inhibited by different alkylphosphates). Therefore, one might expect that other mammalian acetylcholinesterases will possess structures showing structural divergence between these two limiting cases. In this regard, the fetal bovine serum acetylcholinesterase has proven useful. The trend in sequence divergence that we might expect can be seen in examining the N-terminal region of four cholinesterases (Table III). A more complete analysis of this nature should prove very useful in identifying various functional and antigenically cross-reactive regions.

Table III

N-Terminal Sequences of the Cholinesterases

	5	10	15	20	25	30	35	40	45	50	55	60	65
DNA SEQUENCE (1-2-4)	DDHS	ELLV	NTRK	SGKVM	GTR	VPVLSS	HS	SAFLG	IP	FAE	PPV	GNMR	FRPEPKFPWSGVWNA
													STYP
													CHO
TORPEDO (5,65)	D ^D N ^N H ^H S ^S	E ^E LLV ^Q	NTRK	SGKVM	GTR								
TORPEDO (115)	DDHS	ELLV	NTRK	SGKVM	GTR	VPVLSS	HS	SAFLG	IP	FAE	PPV	GNMR	FRPEPKFPWSGVWNA
													STYP
													CHO
BOVINE FETAL SERUM AChE	EGPED	PELLV	MVSG	GELK	GLRL	MAPRGP	VS	SAFLG	IY	FAL	P(I	V	DYR
													EYFF)
HUMAN BuChE	EDD	II	IAT	KNS	SGV	RGM	NLT	VFGG	NVTE	FLG	TPY	LQVPL	GIVLAU)
													CHO

The bovine fetal acetylcholinesterase sequence is from B.P. Doctor (unpublished results). Human butyrylcholinesterase was reported in reference 15.

4. Isolation of c-DNA clones encoding for acetylcholinesterase

Although this portion of the work was initiated and sustained with the support of the National Institutes of Health, the protein chemistry and molecular biological approaches are integrally linked, and it would have been impossible to proceed as rapidly without having both approaches in the same laboratory. Our library screening strategies rely on hybridization with tandem but not overlapping probes, since we initially found that screening with a single probe yielded a very high incidence of false positives. The library was constructed in a γ gt-10 vector and kindly provided by Dr. Toni Claudio at Yale University, New Haven, Connecticut. When sequenced, the false positives were found to be repeating sequences of approximately 500 bp with rather good base matches (14 of the 17 bases in the mixed probe). The tandem probes eliminated this artifact and were preferable to using probes coding for separate peptides. The latter approach will miss short-length sequences. The tandem probe approach usually requires that more amino acid sequence be known, since rather long peptide stretches are usually required to minimize code redundancy in the probes. Positives to both tandem probes were then screened with a probe coding for the N-terminal region. This reduced the number of positives and enhanced the likelihood of obtaining full-length inserts. By this approach we have now obtained 7 inserts which clearly encode for acetylcholinesterase and 13 more candidates. Their lengths and locations of Eco RI sites are detailed in Fig. 3. Only lambda 2-4 has been sequenced. The sequence and sequencing strategies are given in Figs. 4 and 5.

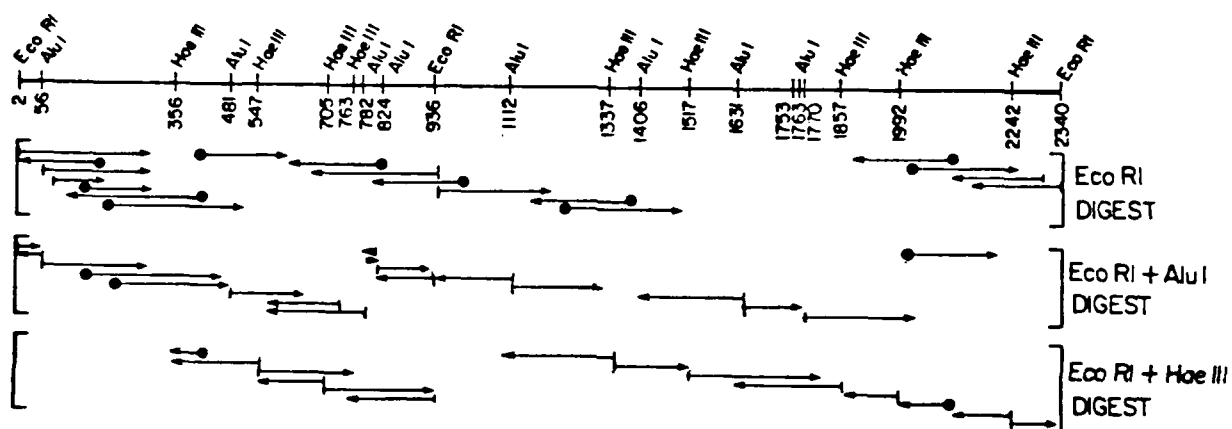


Figure 4: Sequencing strategy for acetylcholinesterase clone AchE-1 (λ2-4). Critical restriction sites (Eco RI, Hae III, Alu I) and sequencing primer sites are shown.

1081 1111 1096 1111 1126
 1111 TCT CCG GAA GAC TTC ATG TCA GGG GTC AAG CTA AGC GTT CCC CAC GGC AAT
 1111 Ser Arg Glu Asp Phe Met Ser Gly Val Lys Leu Ser Val Pro His Ala Asn
 347 1141 1136 1171 1186
 GAC TTA GGG TTG GAC GCT GTC ACB CTA CAG TAC ACA GAG TGG ATG BAT GAC GAC
 347 Leu Gly Leu Asp Ala Val Thr Leu Gln Tyr Thr Asp Trp Met Asp Asp Asn
 1201 1216 1231
 AAT GGT ATA AAG AAC AGA GAT GGA TGG GAC GAC ATC GTA GGG GAC CAC AAC GTC
 1201 Asn Gly Ile Lys Asn Arg Asp Gly Leu Asp Asp Ile Val Gly Asp His Asn Val
 1246 1261 1276 1291
 ATA TGC CCC TTG ATG CAC TTT GTT AAC AAG TAC ACC AAG TTT GGC AAT GGC ACC
 1246 Ile Cys Pro Leu Met His Phe Val Asn Lys Tyr Thr Lys Phe Gly Asn Gly Thr
 1306 1321 1336
 TAC CTG TAC TTC TTC GAC CAC CGA GGC TCA AAC CTD DTG TGG CCG GAG TGG ATG
 1306 Tyr Leu Tyr Phe Phe Asn His Arg Ala Ser Asn Leu Val Trp Pro Gly Trp Met
 1351 1366 1381 1396
 GGC GTC ATC CAC GGC TAT GAG ATT GAG TTC CTT TTC GGG CTG CTT CTG GTG AAG
 1351 Gly Val Ile His Gly Tyr Gly Ile Gly Phe Val Phe Gly Leu Pro Leu Val Lys
 1411 1426 1441 1456
 GAG CTG AAC TAC ACA GCG GAG GAG GAA GCG CTG AGC CCG AAG ATA ATG CAT TAC
 1411 Gly Leu Asn Tyr Thr Ala Gly Gly Ala Leu Ser Arg Arg Ile Met His Tyr
 1471 1486 1501
 TGG GCG ACA TTC GCA AAG ACT GGA AAC CCA AAC GAA CCC CAC TCA CAG GAG AGC
 1471 Trp Ala Thr Phe Ala Lys Thr Gly Asn Pro Asn Gly Pro His Ser Gln Gly Ser
 1516 1531 1546 1561
 AAA TGG CCT CTC TTC ACT ACC AAG GAG CAG AAA TTT ATT GAC CTC AAC ACA GAA
 1516 Lys Trp Pro Leu Phe Thr Thr Lys Gly Gln Lys Phe Ile Asp Leu Asn Thr Gly
 1591 1606
 CCC ATG AAA GTC CAC CAG CGA CTC CCA GTT CAG ATG TGC GTG TTC TGG AAC CAG
 1591 Pro Met Lys Val His Gln Arg Leu Arg Val Gln Met Cys Val Phe Trp Asn Gln
 1621 1636 1651 1666
 TTC CTC CCC AAG CTC CTC AAC GCC ACA GAG ACC ATT GAT GAG GCA GAA CGC CAG
 1621 Phe Leu Pro Lys Leu Leu Asn Ala Thr Gly Thr Ile Asp Gly Ala Gly Arg Gln
 1681 1696 1711 1726
 TGG AAG ACG GAG TTT CAT CCG TGG AGT TCC TAC ATG ATG CAC TGG GAG AAC CAA
 1681 Trp Lys Thr Gly Phe His Arg Trp Ser Tyr Met Met His Trp Lys Asn Gln
 1741 1756 1771 1786
 TTT GAC CAC TAC AGC AGA CAC GAG AGC TGT GCT GAG CTG TGA BCTCTGCTCT GCMGTCGCC
 1741 Phe Asp His Tyr Ser Arg His Gly Ser Cys Ala Gly Leu
 1798 1808 1818 1828 1838 1848 1858
 TGGTGAACCA GAGACGACAG TCCGATATTT ACCAGACACC CAGTCTAGTT CCTGAGAAC CTGCTGCCC
 1798 Trp Gly Asn Asn GAG Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn
 1868 1878 1888 1898 1908 1918 1928
 CTCTGAGCCC CCCCCCCTCA CCCCCCCTCA CCCCCCCTCA CCCCCCCTCA CCCCCCCTCA
 1868 Cys Trp Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn
 1938 1948 1958 1968 1978 1988 1998
 CCGCAGGCTG CCGACCTCTG CTCTGCGACC GTCACTCTGTA AATCTGCGGC ACCAATCTTT CCGCCACCCA
 1938 Cys Gly Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn
 2008 2018 2028 2038 2048 2058 2068
 CTCTGCGACC AGCCCAACTC TCCTCTCTCT CTCTGCGACC ACTACCCCCC TATTACCCAC TCCTGACCTG
 2008 Cys Gly Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn
 2078 2088 2098 2108 2118 2128 2138
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 2078 Cys Gly Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn
 2148 2158 2168 2178 2188 2198 2208
 CCGTCTACCC CCCCCCCTCT ACTCTGTCTCT CCGCAGTACT CCGCTCTCTCT TCATCCCGAG GTTCCCAACT
 2148 Cys Gly Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn
 2218 2228 2238 2248 2258 2268 2278
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 2218 Cys Gly Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn
 2288 2298 2308 2318 2328 2338 2348
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 2288 Cys Gly Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn
 106 111 116 121 126 131 136 141 146 151 156 161 166 171 176 181 186 191 196 201 206 211 216 221 226 231 236 241 246 251 256 261 266 271 276 281 286 291 296 301 306 311 316 321 326 331 336 341 346 351 356 361 366 371 376 381 386 391 396 401 406 411 416 421 426 431 436 441 446 451 456 461 466 471 476 481 486 491 496 501 506 511 516 521 526 531 536 541 546 551 556 561 566 571 576 581 586 591 596 601 606 611 616 621 626 631 636 641 646 651 656 661 666 671 676 681 686 691 696 701 706 711 716 721 726 731 736 741 746 751 756 761 766 771 776 781 786 791 796 801 806 811 816 821 826 831 836 841 846 851 856 861 866 871 876 881 886 891 896 901 906 911 916 921 926 931 936 941 946 951 956 961 966 971 976 981 986 991 996 1001 1006 1011 1016 1021 1026 1031 1036 1041 1046 1051 1056 1061 1066 1071 1076 1081 1086 1091 1096 1101 1106 1111 1116 1121 1126 1131 1136 1141 1146 1151 1156 1161 1166 1171 1176 1181 1186 1191 1196 1201 1206 1211 1216 1221 1226 1231 1236 1241 1246 1251 1256 1261 1266 1271 1276 1281 1286 1291 1296 1301 1306 1311 1316 1321 1326 1331 1336 1341 1346 1351 1356 1361 1366 1371 1376 1381 1386 1391 1396 1401 1406 1411 1416 1421 1426 1431 1436 1441 1446 1451 1456 1461 1466 1471 1476 1481 1486 1491 1496 1501 1506 1511 1516 1521 1526 1531 1536 1541 1546 1551 1556 1561 1566 1571 1576 1581 1586 1591 1596 1601 1606 1611 1616 1621 1626 1631 1636 1641 1646 1651 1656 1661 1666 1671 1676 1681 1686 1691 1696 1701 1706 1711 1716 1721 1726 1731 1736 1741 1746 1751 1756 1761 1766 1771 1776 1781 1786 1791 1796 1801 1806 1811 1816 1821 1826 1831 1836 1841 1846 1851 1856 1861 1866 1871 1876 1881 1886 1891 1896 1901 1906 1911 1916 1921 1926 1931 1936 1941 1946 1951 1956 1961 1966 1971 1976 1981 1986 1991 1996 2001 2006 2011 2016 2021 2026 2031 2036 2041 2046 2051 2056 2061 2066 2071 2076 2081 2086 2091 2096 2101 2106 2111 2116 2121 2126 2131 2136 2141 2146 2151 2156 2161 2166 2171 2176 2181 2186 2191 2196 2201 2206 2211 2216 2221 2226 2231 2236 2241 2246 2251 2256 2261 2266 2271 2276 2281 2286 2291 2296 2301 2306 2311 2316 2321 2326 2331 2336 2341 2346 2351 2356 2361 2366 2371 2376 2381 2386 2391 2396 2401 2406 2411 2416 2421 2426 2431 2436 2441 2446 2451 2456 2461 2466 2471 2476 2481 2486 2491 2496 2501 2506 2511 2516 2521 2526 2531 2536 2541 2546 2551 2556 2561 2566 2571 2576 2581 2586 2591 2596 2601 2606 2611 2616 2621 2626 2631 2636 2641 2646 2651 2656 2661 2666 2671 2676 2681 2686 2691 2696 2701 2706 2711 2716 2721 2726 2731 2736 2741 2746 2751 2756 2761 2766 2771 2776 2781 2786 2791 2796 2801 2806 2811 2816 2821 2826 2831 2836 2841 2846 2851 2856 2861 2866 2871 2876 2881 2886 2891 2896 2901 2906 2911 2916 2921 2926 2931 2936 2941 2946 2951 2956 2961 2966 2971 2976 2981 2986 2991 2996 3001 3006 3011 3016 3021 3026 3031 3036 3041 3046 3051 3056 3061 3066 3071 3076 3081 3086 3091 3096 3101 3106 3111 3116 3121 3126 3131 3136 3141 3146 3151 3156 3161 3166 3171 3176 3181 3186 3191 3196 3201 3206 3211 3216 3221 3226 3231 3236 3241 3246 3251 3256 3261 3266 3271 3276 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4281 4286 4291 4296 4301 4306 4311 4316 4321 4326 4331 4336 4341 4346 4351 4356 4361 4366 4371 4376 4381 4386 4391 4396 4401 4406 4411 4416 4421 4426 4431 4436 4441 4446 4451 4456 4461 4466 4471 4476 4481 4486 4491 4496 4501 4506 4511 4516 4521 4526 4531 4536 4541 4546 4551 4556 4561 4566 4571 4576 4581 4586 4591 4596 4601 4606 4611 4616 4621 4626 4631 4636 4641 4646 4651 4656 4661 4666 4671 4676 4681 4686 4691 4696 4701 4706 4711 4716 4721 4726 4731 4736 4741 4746 4751 4756 4761 4766 4771 4776 4781 4786 4791 4796 4801 4806 4811 4816 4821 4826 4831 4836 4841 4846 4851 4856 4861 4866 4871 4876 4881 4886 4891 4896 4901 4906 4911 4916 4921 4926 4931 4936 4941 4946 4951 4956 4961 4966 4971 4976 4981 4986 4991 4996 5001 5006 5011 5016 5021 5026 5031 5036 5041 5046 5051 5056 5061 5066 5071 5076 5081 5086 5091 5096 5101 5106 5111 5116 5121 5126 5131 5136 5141 5146 5151 5156 5161 5166 5171 5176 5181 5186 5191 5196 5201 5206 5211 5216 5221 5226 5231 5236 5241 5246 5251 5256 5261 5266 5271 5276 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6281 6286 6291 6296 6301 6306 6311 6316 6321 6326 6331 6336 6341 6346 6351 6356 6361 6366 6371 6376 6381 6386 6391 6396 6401 6406 6411 6416 6421 6426 6431 6436 6441 6446 6451 6456 6461 6466 6471 6476 6481 6486 6491 6496 6501 6506 6511 6516 6521 6526 6531 6536 6541 6546 6551 6556 6561 6566 6571 6576 6581 6586 6591 6596 6601 6606 6611 6616 6621 6626 6631 6636 6641 6646 6651 6656 6661 6666 6671 6676 6681 6686 6691 6696 6701 6706 6711 6716 6721 6726 6731 6736 6741 6746 6751 6756 6761 6766 6771 6776 6781 6786 6791 6796 6801 6806 6811 6816 6821 6826 6831 6836 6841 6846 6851 6856 6861 6866 6871 6876 6881 6886 6891 6896 6901 6906 6911 6916 6921 6926 6931 6936 6941 6946 6951 6956 6961 6966 6971 6976 6981 6986 6991 6996 7001 7006 7011 7016 7021 7026 7031 7036 7041 7046 7051 7056 7061 7066 7071 7076 7081 7086 7091 7096 7101 7106 7111 7116 7121 7126 7131 7136 7141 7146 7151 7156 7161 7166 7171 7176 7181 7186 7191 7196 7201 7206 7211 7216 7221 7226 7231 7236 7241 7246 7251 7256 7261 7266 7271 7276 7281 7286 7291 7296 7301 7306 7311 7316 7321 7326 7331 7336 7341 7346 7351 7356 7361 7366 7371 7376 7381 7386 7391 7396 7401 7406 7411 7416 7421 7426 7431 7436 7441 7446 7451 7456 7461 7466 7471 7476 7481 7486 7491 7496 7501 7506 7511 7516 7521 7526 7531 7536 7541 7546 7551 7556 7561 7566 7571 7576 7581 7586 7591 7596 7601 7606 7611 7616 7621 7626 7631 7636 7641 7646 7651 7656 7661 7666 7671 7676 7681 7686 7691 7696 7701 7706 7711 7716 7721 7726 7731 7736 7741 7746 7751 7756 7761 7766 7771 7776 7781 7786 7791 7796 7801 7806 7811 7816 7821 7826 7831 7836 7841 7846 7851 7856 7861 7866 7871 7876 7881 7886 7891 7896 7901 7906 7911 7916 7921 7926 7931 7936 7941 7946 7951 7956 7961 7966 7971 7976 7981 7986 7991 7996 8001 8006 8011 8016 8021 8026 8031 8036 8041 8046 8051 8056 8061 8066 8071 8076 8081 8086 8091 8096 8101 8106 8111 8116 8121 8126 8131 8136 8141 8146 8151 8156 8161 8166 8171 8176 8181 8186 8191 8196 8201 8206 8211 8216 8221 8226 8231 8236 8241 8246 8251 8256 8261 8266 8271 8276 8281 8286 8291 8296 8301 8306 8311 8316 8321 8326 8331 8336 8341 8346 8351 8356 8361 8366 8371 8376 8381 8386 8391 8396 8401 8406 8411 8416 8421 8426 8431 8436 8441 8446 8451 8456 8461 8466 8471 8476 8481 8486 8491 8496 8501 8506 8511 8516 8521 8526 8531 8536 8541 8546 8551 8556 8561 8566 8571 8576 8581 8586 8591 8596 8601 8606 8611 8616 8621 8626 8631 8636 8641 8646 8651 8656 8661 8666 8671 8676 8681 8686 8691 8696 8701 8706 8711 8716 8721 8726 8731 8736 8741 8746 8751 8756 8761 8766 8771 8776 8781 8786 8791 8796 8801 8806 8811 8816 8821 8826 8831 8836 8841 8846 8851 8856 8861 8866 8871 8876 8881 8886 8891 8896 8901 8906 8911 8916 8921 8926 8931 8936 8941 8946 8951 8956 8961 8966 8971 8976 8981 8986 8991 8996 9001 9006 9011 9016 9021 9026 9031 9036 9041 9046 9051 9056 9061 9066 9071 9076 9081 9086 9091 9096 9101 9106 9111 9116 9121 9126 9131 9136 9141 9146 9151 9156 9161 9166 9171 9176 9181 9186 9191 9196 9201 9206 9211 9216 9221 9226 9231 9236 9241 9246 9251 9256 9261 9266 9271 9276 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The insert begins by encoding a 16-amino acid leader peptide, extends through bases encoding the 575 amino acids of the processed protein, and contains another 571 site bases in a 3' noncoding region (fig. 5). It does not contain a poly A termination site or a canonical poly A termination signal, which suggests that this 3' region is not complete (4). Clone 2-4 was sequenced in M-13 by the dideoxy method. Protein sequence, again, provided confirmation that the selected open reading frame was correct and did not divaricate because of a skipped base. In addition, the protein sequence enabled us to establish that the clone likely encoded for the 11S species. We are sequencing the other clones based on initial findings, and are optimistic that we have found a clone for another acetylcholinesterase species. There is one caveat: The fact that all clones end in Eco RI sites suggests incomplete methylation in the library preparation. Clones 1-1, 1-9 and 1-10 are probably identical but reflect another gene of acetylcholinesterase. They are being sequenced. Clone 1-9, which is nearly 2.9 kb in length, is our candidate for obtaining complete 5' and 3' noncoding regions. Clone 2-9 is probably a shortened version of 1-9, terminating at the Eco RI site. In short, valuable information will continue to accrue as we compare cDNA inferred sequences with actual protein sequences. Accordingly, the combination of molecular biology and protein chemistry should enable us to identify all of the structural polymorphisms in Torpedo acetylcholinesterase.

5. Isolation of additional acetylcholinesterase clones

The original clone (AchE-1) was used to select additional clones in the λ -gt-10 cDNA library. In all, 117 positive recombinants were identified and isolated. Since the library was amplified, all of the recombinants do not reflect unique inserts, but on the basis of insert size we have been able to identify at least 15 unique clones. Unfortunately, none of the clones encode a sequence we can identify as the 5.6S species. This has also been confirmed with initial data where we have employed a probe which spans both sides of the putative exon-intron exon splice site which gives rise to the message for the 11S enzyme.

Nevertheless, three clones yielding unique information have been identified:

a. AchE-11. This clone is identical in the open reading frame to AchE-1, but contains an extended 5' region, giving us the entire 21 amino acid leader sequence and a sequence of approximately 140 base pairs in the 5' noncoding region.

b. AchE-14. This clone is identical in the open reading frame to AchE-1 and AchE-11 but contains a deletion in the 5' noncoding region. This appears to be an alternative sequence in this region. The deletion point starts at a bona fide splice site.

c. AchE-16. This clone, while short, extends the sequence of AchE-1 in the 3' direction for another 80 bp.

6. Determination of the secondary structure of acetylcholinesterase

A major portion of the third year was directed to this important project. The procedures involved tryptic and CNBr digestion of the unreduced protein followed by separation of the individual peptides on HPLC. Peptides not detected in the reduced enzyme profiles were separated. They were reduced and carboxymethylated with [^{14}C]iodoacetate.

The reduced peptides were analyzed for radioactivity and sequence. Once free, SH could be detected by labeling with monobromobimane in the absence of reduction. This was Cys 231 . Three pairs of disulfide-bonded peptides were identified: loop A, Cys 67 -Cys 94 ; loop B, Cys 254 -Cys 265 ; loop C, Cys 402 -Cys 521 . Cys 572 was linked to an identical peptide. This disulfide, three residues from the C-terminal position, forms the inter-subunit cross-link with homologous subunits. The three intrachain disulfide loops A, B and C are conserved in thyroglobulin and butyrylcholinesterase, demonstrating that these proteins are likely to have identical folding patterns. The disulfide bonding pattern and homology with thyroglobulin are shown in Figs. 6 and 7.

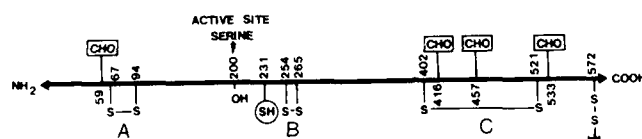
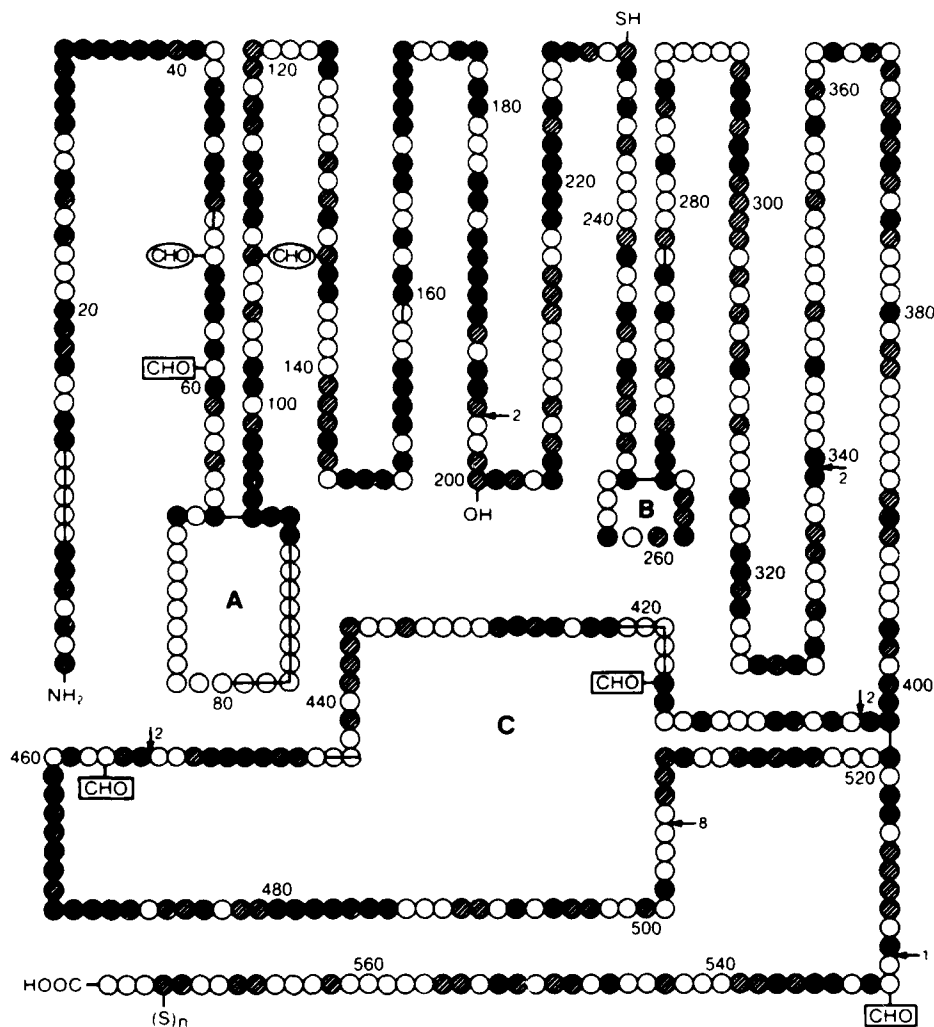


Figure 6. Localization of the sulfhydryl groups and glycosylation sites in acetylcholinesterase. CHO indicates carbohydrate attachment sites, disulfide bonds are connected by solid lines (S-----S), OH indicates the active site serine, and SH indicates a free sulfhydryl group; NH₂ and COOH indicate the amino- and carboxy termini, respectively. The three disulfide loops are designated A, B, and C. Numbers denote the position of each residue based on the cDNA-deducted sequence.

Acetylcholinesterase: Disulfide Bonds in 11S Acetylcholinesterase

Figure 7

Schematic representation of the homology and common secondary structure between acetylcholinesterase (residues 1-575) and thyroglobulin (residues 2182-2750). Numbering is based on the deduced sequence of acetylcholinesterase. Loops created by disulfide bonds are indicated by A, B, and C. ● indicates identical residues; ○ indicates conservative changes; OH indicates the active site serine; SH, the free sulfhydryl group; [CHO], AChE carbohydrate attachment sites; (S)_n, disulfide link to a secondary AChE monomer. Homologous residues with thyroglobulin are as indicated. Arrows denote residue gaps in acetylcholinesterase; the number indicates the number of residues missing; - indicates residue gaps in thyroglobulin sequence. (CHO), thyroglobulin carbohydrate attachment sites. Conservative changes reflect amino acid substitutions that could be encoded by a single base difference.



7. General aspects of acetylcholinesterase structure deduced from amino acid and nucleotide sequencing

All of the above data enable us to arrive at the following conclusions:

a. Acetylcholinesterase contains a hydrophobic leader sequence (residues -21 to 0) but contains no other hydrophobic domains which are candidates for membrane-spanning regions. Thus it is likely to be an exported protein and its membrane attachment site(s) arises as a consequence of posttranslational modifications.

b. The active center serine is at residue 200. The N-terminal location contrasts with the serine proteases of similar size that function in the clotting cascade (i.e., factor IX and prothrombin).

c. No significant global or local homology is found with the acetylcholine receptor.

d. Although acetylcholinesterase is closely homologous to human butyrylcholinesterase, no significant global homology and very limited local homology are found with other serine proteases. The largest local homology is seen with liver aliesterase and the carboxylesterases.

e. Substantial homology is found between acetylcholinesterase and thyroglobulin in their C-terminal regions (acetylcholinesterase residues 1-575; thyroglobulin residues 2168-2750). Six of the eight cysteines are conserved, suggesting a similar folding pattern for the two macromolecules. The region between residues 160 and 190 shows greater than 60% identity.

f. The homology with thyroglobulin shows that six conserved cysteines are the ones forming the three disulfide loops in acetylcholinesterase. The SH involved in the intersubunit disulfide linkage at 572 and the free cysteine Cys 231 are not conserved. This striking comparison shows that the secondary structure and folding pattern of acetylcholinesterase and thyroglobulin are virtually identical.

g. The homology between Torpedo acetylcholinesterase and human butyrylcholinesterase (53% residue identity) (15) reveals substantial conservation among the cholinesterases. A comparison of Torpedo acetylcholinesterase with a fetal bovine serum acetylcholinesterase shows about 60% residue identity. The latter enzyme is being sequenced by B.P. Doctor and colleagues. Substantial homology (~30% residue identity) with certain other esterases (microsomal esterase, *Drosophila* Est 6 and *Dictyostelium* D-3) have also been reported.

8. Ongoing studies

Several studies have been initiated but results are still too preliminary to give a detailed account. These studies include:

a. Detection of antigenic regions within the acetylcholinesterase molecule. For this purpose several monoclonal antibodies previously mentioned are being employed.

b. Genomic cloning of acetylcholinesterase.

c. Detection of regions within the primary structure in the hydrophobic dimer forms of acetylcholinesterase which are unique and differ from the asymmetric forms.

d. Analysis of carbohydrate structures of the various forms of acetylcholinesterase.

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